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CORRECTION OF QUENCHING ERRORS IN ANALYTICAL FLUORIMETRY THROUG--ETC(U)
MAY 80 G M HIEFTJE, G R HAUGEN N00014-76-C-0838
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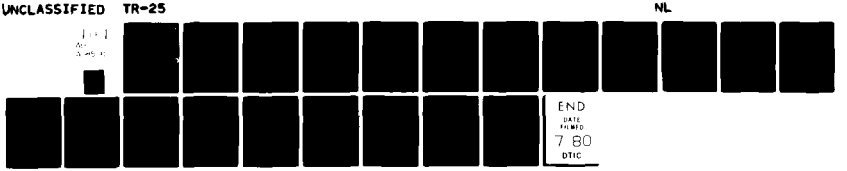

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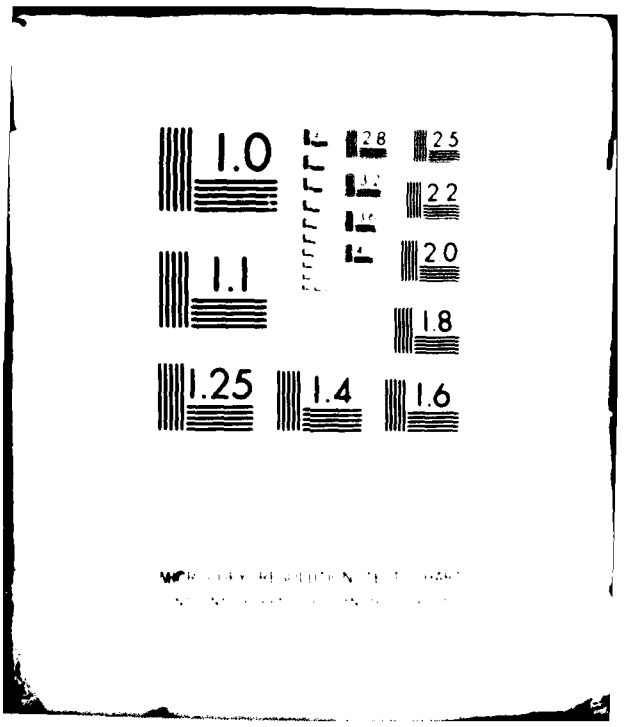
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CORRECTION OF QUENCHING ERRORS IN ANALYTICAL FLUORIMETRY THROUGH USE OF TIME RESOLUTION

by

Gary M. Hieftje and Gilbert R. Haugen

Prepared for Publication

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Indiana University

Department of Chemistry

Bloomington, Indiana 47405

May 27, 1980

ABSTRACT

Varying concentrations of quenching agents can cause serious errors in analytical fluorimetry. The origin of these errors is an unexpected change in the quantum efficiency for the observed luminescence. In this paper, it is recalled that quantum efficiency can be expressed as the ratio of an observed luminescence decay time to the decay time which would be observed in the absence of quenchers. Because this latter quantity is a constant for any particular fluorophore, quantum efficiency variations can be compensated through measurement of the decay time. For such measurements, the time-correlated single photon technique was employed and measured luminescence values were taken both from averaged photon count rates and from integrated fluorescence decay plots. Division of these values by measured luminescence lifetimes produced values which were independent of quencher concentration. Systems studied were quinine bisulfate quenched with chloride ion and 1-pyrenebutyric acid quenched by iodide.

INTRODUCTION

Fluorimetry provides one of the most sensitive means for the detection of many species of clinical, environmental, or forensic interest. However, fluorimetric analysis, unlike absorption methods, can suffer interferences from foreign species which quench excited analyte molecules. Although a number of mechanisms for such quenching action exist, they produce the same result--a reduced quantum efficiency and a consequent loss in fluorescence intensity. Because the concentration of the quenching agent is ordinarily unknown, correction for quenching is difficult or impossible; consequently, strong measures are taken to exclude quenchers from the analytical medium or to control carefully their concentration.

In the present paper, an alternative approach to overcoming quenching errors is explored. In this approach, quantum efficiency is indirectly measured by monitoring a sample's luminescence lifetime; correction for quenching then simply involves division of the measured fluorescence intensity by the observed lifetime.

Fundamentally, the new approach is quite straightforward. It is well known that the fluorescence intensity from the sample (F) is related to sample concentration (c) through the quantum efficiency (Φ) and a proportionality constant K.

$$F = K\Phi c. \tag{1}$$

In turn, quantum efficiency is simply the ratio of the intrinsic decay rate of an excited state (k_F) to the sum of the decay rates of all processes which depopulate the state (Σk):

$$\Phi = k_F/\Sigma k \tag{2}$$

Conveniently, the demoninator in equation 2 is simply the reciprocal of the decay time for the sample under examination (τ_m), whereas the numerator is a reciprocal of the intrinsic lifetime of the fluorophore (τ_F):

$$\Phi = \tau_m/\tau_F \tag{3}$$

Combining equations 1 and 3 yields

$$F/\tau_m = k'c \tag{4}$$

where k' contains both K from equation 1 and τ_F.

From equation 4, it should be possible to obtain a quantity which is independent of sample quantum efficiency simply by dividing a measured fluorescence signal (F) by the measured luminescence lifetime (τ_m). In the present investigation, these measurements were carried out for two different fluorophore/quencher combinations using a time-correlated single photon technique for measurement of both luminescence lifetimes and fluorescence intensities. Although the precision of the resulting corrected values was limited to approximately 4% by errors in the lifetime measurement procedure, it was found possible to correct for quenching effects in samples whose fluorescence had been quenched by 95%. Although the procedure is not able to overcome errors caused by <u>all</u> kinds of quenching processes, it is expected to be important in situations involving diffusional quenching and changes in intersystem crossing rates.

EXPERIMENTAL

Two different instrumental configurations were employed for both the measurement of fluorescence intensities and luminescence decay curves. These configurations will be described separately.

System 1. For the lifetime measurements, a commercial time-correlated single photon fluorimeter (ORTEC model 9200), modified for high data acquisition rates (1) was employed. The pulsed excitation source was run in air at 20 psig at a frequency of 25 KHz. For excitation wavelength selection, an interference filter peaked at 334 nm and having a bandwidth of 11 nm was used; emission was selected by a cutoff filter (Corning #0-52). The photomultipler (RCA 8850) was uncooled and operated at a voltage of 2700 V.

Fluorescence intensities were measured using a computer-compatible system described previously (2). An excitation wavelength of 340 nm and emission wavelengths 375 and 395 nm were employed. Analog data collection was accomplished by chopping of the exciting radiation (4 KHz) and tuned, lock-in amplification. A 4-pole Bessel active filter with a bandwidth of 0.2 Hz smoothed the output of the lock-in amplifier. The S-20 photomultiplier (Hamamatsu 65TUVP) was operated at 2060 volts.

System 1 was utilized for examining the correction of 1-pyrenebutyric acid quenched by potassium iodide (3).

System 2. A second instrumental array was employed in an attempt to extract all necessary data for corrected luminescence measurements from a single device. Unfortunately, such capability does not exist in conventional time-correlated single photon instruments because of large fluctuations in source intensity. Attempts were made to overcome this limitation through

counting of lamp pulses during the elapsed measurement interval and integrating the resulting luminescence decay curve. However, precision of these measurements was far poorer than could be obtained from a conventional luminescence spectrometer.

To overcome these difficulties, the high-pressure air-discharge lamp was replaced by a much stabler source consisting of a synchronously pumped, cavity-dumped, frequency-doubled dye laser. The laser was a commercial unit (models 171, 375, and 344, Spectra Physics, Mountain View, CA) operated with rhodamine 6-G as the dye. A pump wavelength of 514.5 nm and dye emission wavelength of 617 nm were utilized, resulting in an excitation pulse train at 308.5 nm. The frequency doubling crystal (Cleveland Crystal Co.) was KDP. This arrangement resulted in a primary beam having a power of approximately 17 mW and consisting of a train of 20 ps pulses at a frequency of 0.8 MHz. These primary pulses are calculated to have a peak power of approximately 1 KW and result in the production of frequency-doubled pulses of approximately 2 W peak power.

To serve as a trigger for the TAC of the correlation fluorimeter, a PIN photodiode registered the arrival of a fraction of the primary beam pulses. The primary and doubled beams were separated using a simple prism arrangement. The only other change from system 1, described above, is the use of a different cut-off filter (Schott #KV389) for separating the fluorescence radiation from background. A similar arrangement has been described previously (1).

With this stable pulsed source, it became possible to measure fluorescence intensity directly from recorded decay curves. Two approaches for this mea-

surement were examined. The first approach involved simply measuring the average anode pulse rate from the photomultiplier detector over a 10-second time period. The second approach utilized the integral of the processed luminescence decay curve as an indication of fluorescence intensity. This integral was readily obtained and could be directly read out from the commercial multichannel pulse-height analyzer employed in these studies.

The second system was employed to study the quenching of quinine bisulfate by chloride ion.

Reagents and chemicals. Both quenching systems have been discussed in some detail in the literature and solutions were prepared in accordance with instructions in that publication (3).

1-pyrenebutyric acid stock solutions were prepared in 0.01 M KOH and combined with varying amounts of iodide ion (as KI) in Tris buffer solutions (pH 8.0). KI was stabilized by the addition of $Na_2S_2O_3$. Solutions were made to have a range of lifetimes between 18 and 115 ns, the latter solution being totally unquenched. No attempt was made to exclude oxygen from the sample solution, since the technique should also correct for intersystem crossing rates induced by the presence of dissolved O_2.

Quinine bisulfate solutions were prepared in 0.1 N H_2SO_4 and were combined with varying amounts of chloride ion (added as NaCl) to yield a group of solutions having expected lifetimes between 1.5 and 18.9 ns.

RESULTS AND DISCUSSION

In general, results were similar for the studies on 1-pyrenebutyric acid and those on quinine bisulfate. However, instrumental system 2 seemed

to perform better than system 1 and was employed for all subsequent investigations. Consequently, we will concentrate on results obtained with this more elegant (but more complex) arrangement.

It was verified initially that quenching in both systems occurred by diffusion-controlled processes; Stern-Volmer plots for quinine bisulfate are shown in Figure 1. In Figure 1 are shown the results for both the anode pulse rate (curve a) and pulse-height spectrum integral (curve b) methods of monitoring fluorescence intensity. Examination of these curves indicates the slightly higher precision of the latter approach, an unsurprising result in view of the larger number of data points utilized by the technique.

The influence of quencher (chloride ion) concentration on quinine bisulfate luminescence intensity is shown in Figures 2a and 2b. Again, these two parts correspond to the different methods for monitoring fluorescence intensity. The lower curve in each figure reflects the drastic change in fluorescence which would be noted if no correction is made. In contrast, the upper, nearly flat line shows the improvement which is obtained by dividing the measured fluorescence intensity for each sample by its measured luminescence lifetime. All curves are least-squares fit to recorded data. For the lifetime-corrected values, relative standard deviations of 5.9% and 4.0% were obtained for, respectively, the anode pulse rate and pulse height integral techniques for monitoring fluorescence intensity. Essentially all this error can be ascribed to uncertainties in the measurement of luminescence decay times.

One of the limitations in the time-correlated single photon technique for the measurement of luminescence lifetimes is that data acquisition times

are long, being limited by the necessity to avoid pulse pileup in the detection system. Although there are ways to minimize this limitation (1,4), there is often a tendency to push data collection limits to improve acquisition time. Consequently, it was decided to examine the influence of unusually high anode pulse rates on the ability to correct for quantum efficiency changes.

The results of these investigations are shown in Figures 3a and 3b. Although the Stern-Volmer plots were linear for both methods of monitoring fluorescence intensity, even at high anode pulse rates, errors in measuring accurate luminescence lifetimes increased dramatically. At high anode pulse rates, fluorescence decay curves appear skewed toward shorter times; unless anode rates are matched exactly for samples having different degrees of quenching, decay curves yield erroneous lifetime values. Moreover, anode pulse overlap would result in a loss of integrated counts in the pulse height spectrum and in the measurement of anode pulse rate, thereby yielding incorrect values for fluorescence intensity. In the present experiment, anode pulse rates were measured with a high-frequency counter, so error in those values was minimized. In contrast, the pulse height spectrum integral was obtained directly from the time-correlated single photon counting apparatus, which had a relatively long dead time. Consequently, data scatter in the corrected values obtained with this latter technique was much greater (relative standard deviation = 7.9% in Figure 3b).

In summary, it has been shown that errors ordinarily caused by quantum efficiency variations in analytical fluorimetry can be overcome through use of time resolution. To perform the correction, one need only monitor simultaneously or consecutively the fluorescence intensity and luminescence

lifetime for each sample. Ratioing these two values then provides a measure of concentration which is independent of collisional quenching processes. Conveniently, both intensity and lifetime can be monitored using the time-correlated single photon technique, if a source having stable pulse amplitudes is employed. However, for conventional instruments, it will be necessary to utilize an independent method for monitoring fluorescence signals.

In this correction procedure, as in most others using the time correlated method, errors in measured lifetimes arise if fluorescence photons are detected at excessively high frequencies. These errors have greatest effect in the present measurements if fluorescence intensity is monitored through use of the pulse height spectrum integral; their effect is minimized if the anode pulse rate is measured directly.

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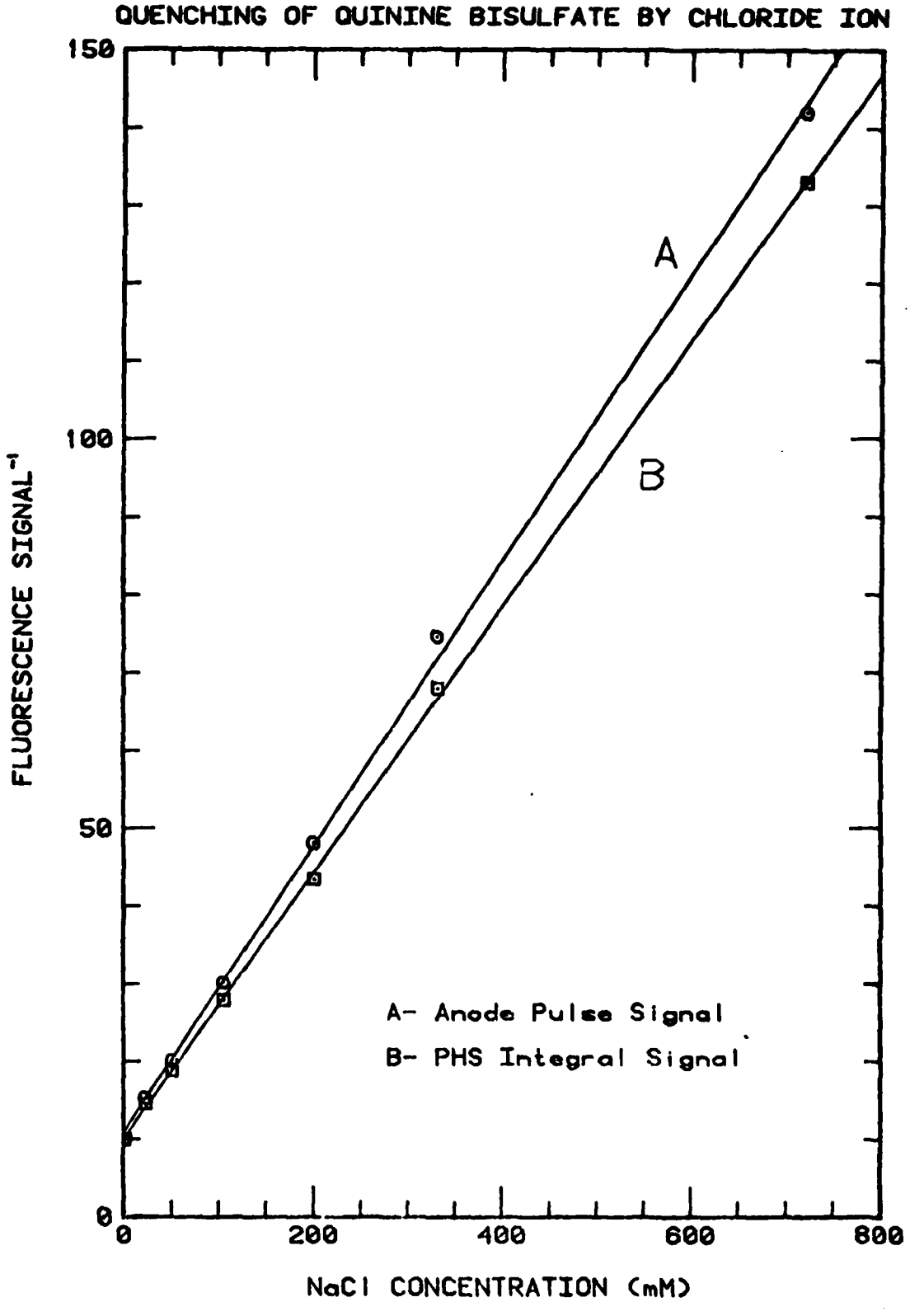
Figure Captions

Figure 1. Stern-Volmer plots for the quenching of quinine bisulfate by chloride ion. Curve A - fluorescence intensity monitored through average anode pulse rate. B - fluorescence intensity obtained by integration of luminescence decay curve (pulse-height spectrum).

Figure 2. Correction of measured fluorescence by ratioing with observed luminescence lifetime. Bottom curve in each figure is directly monitored fluorescence; upper plot reveals corrected values. Part A employs anode pulse rate to indicate fluorescence intensity; part B utilizes integral of pulse height spectrum.

Figure 3. Effect of high frequency of detected fluorescence photons on validity of quenching correction method. All parameters similar to those in Figure 2.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B- PHS Integral Signal
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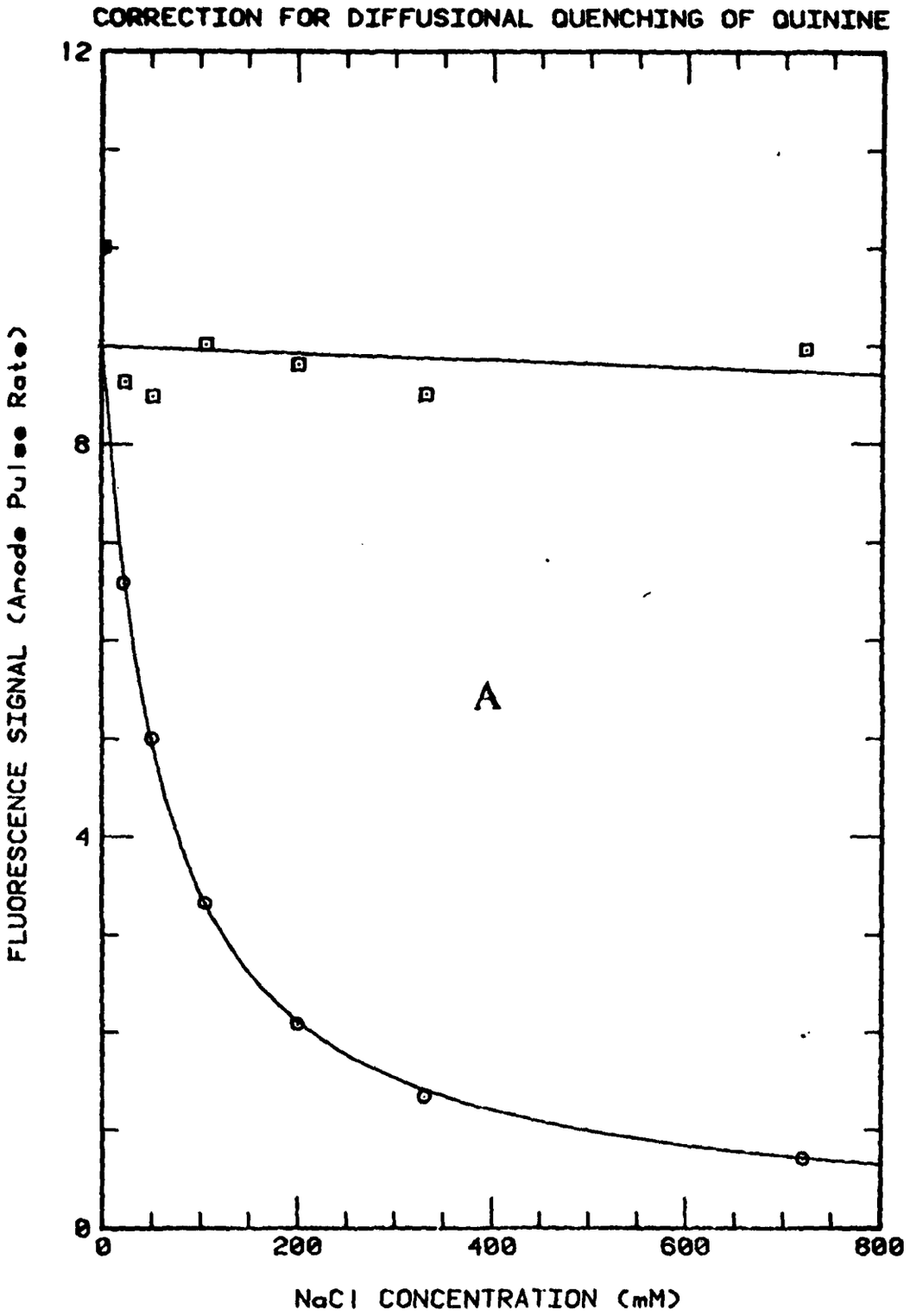
NaCl CONCENTRATION (mM)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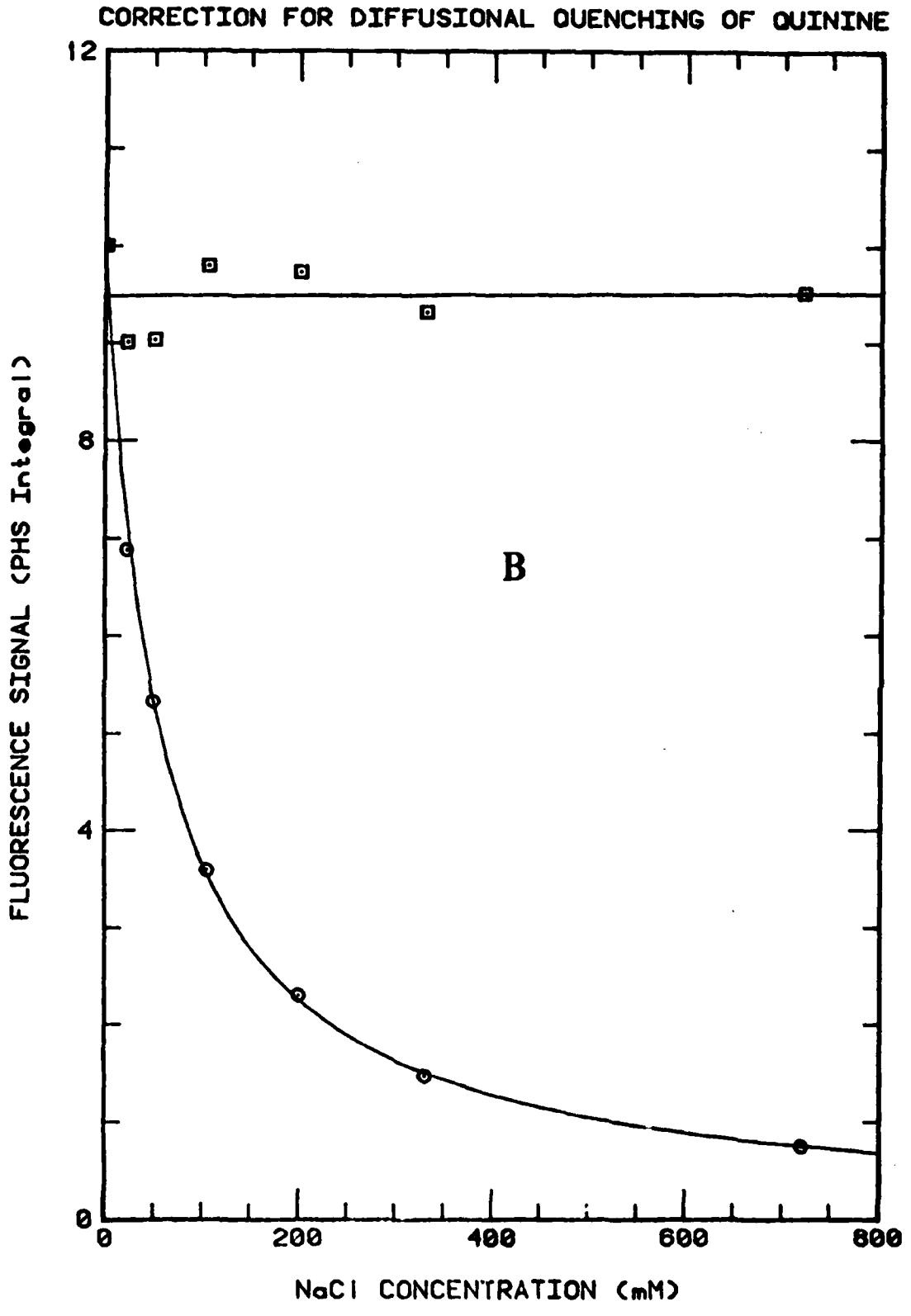
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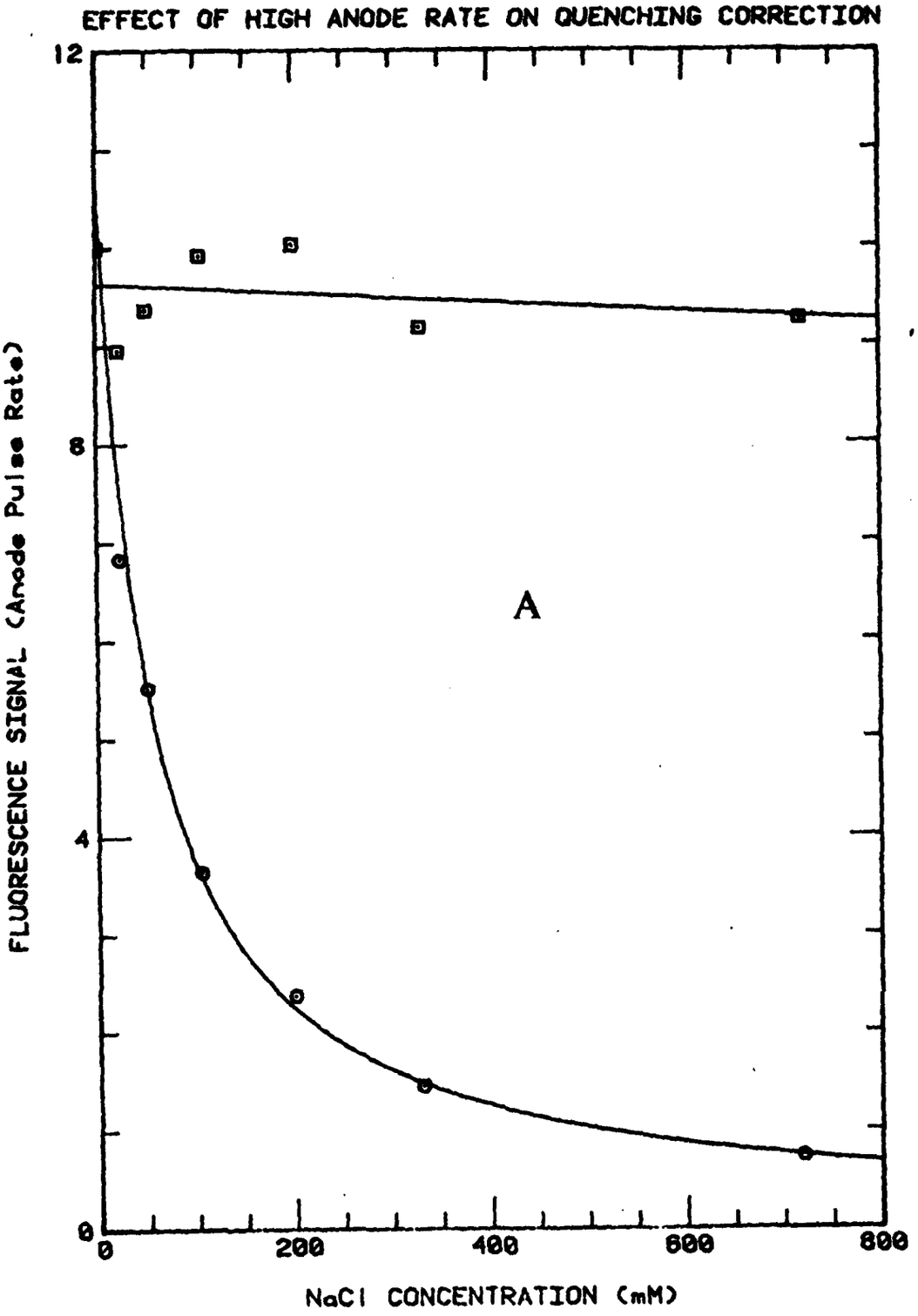
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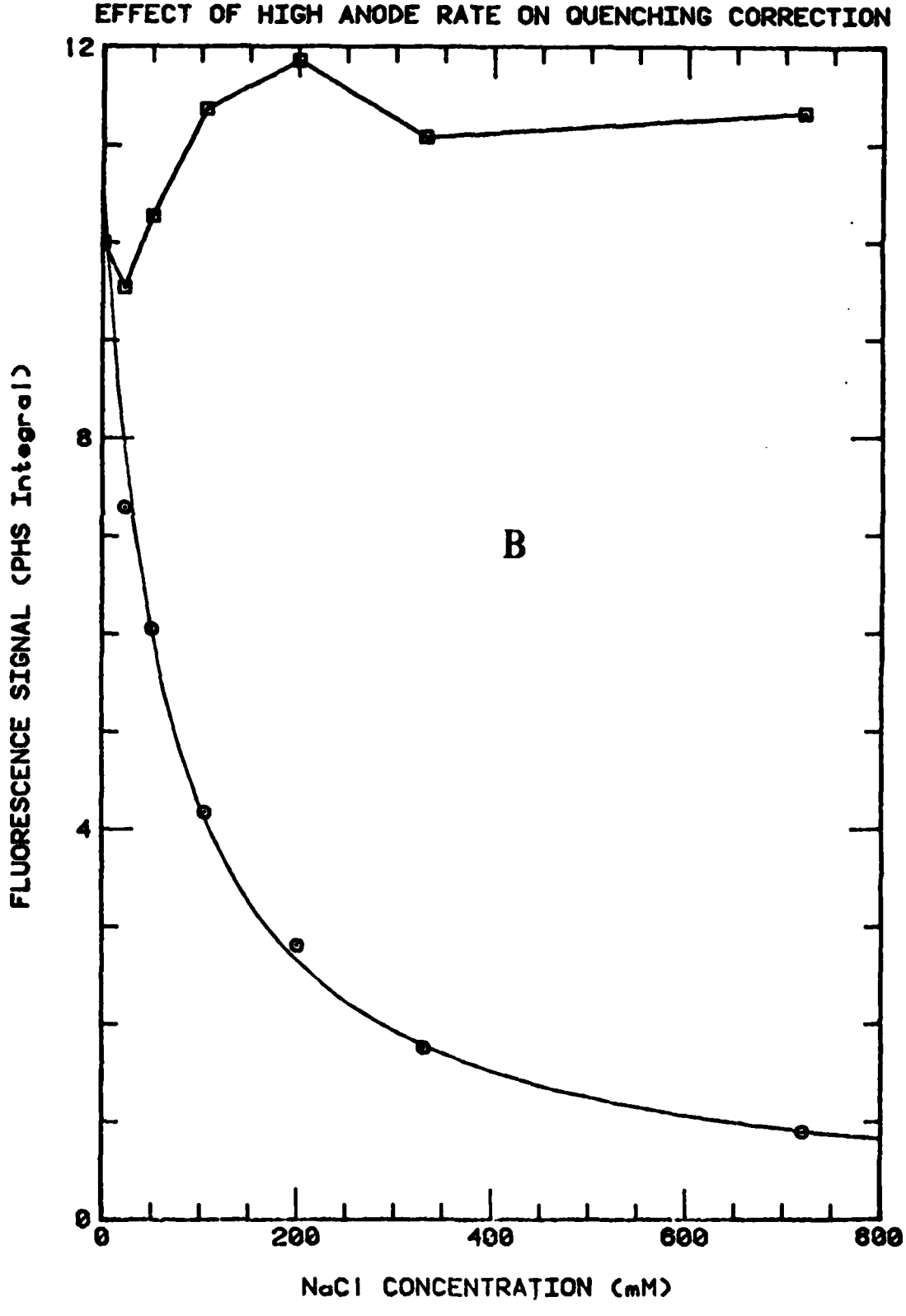
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